

**Instruction of Use:**

**I. Intended Use**

Vita-Assay™ AN6W series is for the functional enrichment of viable tumor progenitor and other rare stem cells from whole blood, lymph, bone marrow and other bodily fluids. Enriched cells can be analyzed by microscopy, flow cytometry and PCR. Enriched cells can also be cultured in the same device for functional analyses of viable tumor progenitor and other rare cells, including immuno-phenotyping, counting, genomic and expression profiling, as well as CTC drug sensitivity (CDS) testing.

- For research use only.
- Not for diagnostic or therapeutic use.



**Product Numbers:**

<b>102.01N (Vita-Assay™ AN6W)</b>	<b>102.02R (Vita-Assay™ AR6W)</b>	<b>102.03G (Vita-Assay™ AG6W)</b>
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<b>Product Specifications</b>	
Each kit Contains:	1 plate of Vita-Assay™ coated with the plain CAM film (N), red fluorescent CAM (R) or green fluorescent CAM (G). 1 tube of Cell Releasing CAM Enzyme. *
No. Cells Processed:	6 samples of 1-10 x 1,000,000 nucleated cells, i.e., nucleated cells in 1-mL of whole blood shall be loaded into one well of the 6-well plate. One Vita-Assay™ RCE AN6W plate can accommodate a total of 6-mL whole blood. Recommended to use with the nucleated cells prepared by red blood cell lysis of whole blood.
Starting Sample:	Whole blood, bone marrow or nucleated cells.
Downstream Applications:	Fluorescence microscopy, Flow cytometry, FISH, etc*; Quantitative RT-PCR, DNA microarray, SAGE, proteomics and genomics**.

Vita-Assay™ AN6W series is for the functional enrichment of viable tumor progenitor and other rare stem cells from whole blood, lymph, bone marrow and other bodily fluids. Enriched cells can be analyzed by microscopy, flow cytometry and PCR. Enriched cells can also be cultured in the same device for functional analyses of viable tumor progenitor and other rare cells, including immuno-phenotyping, counting, genomic and expression profiling, as well as CTC drug sensitivity (CDS) testing. Usually, \* CAM-avid cells released from CAM scaffolds by Cell Releasing CAM Enzyme plus Trypsin/EDTA shall be concentrated to a small volume, i.e., 100 µL PBS containing the enriched cell fraction. The 100 µL cell suspension can be processed for cellular analyses such as fluorescence microscopy, FISH and flow cytometry. \*\* CAM-avid cells released from CAM scaffolds by Cell Releasing CAM Enzyme plus Trypsin/EDTA

shall be concentrated to a small volume, 10  $\mu$ L PBS containing the enriched cell fraction. The 10  $\mu$ L cell suspension can be lysed for protein, mRNA or DNA isolation, followed by proteomics, quantitative RT-PCR, DNA microarray, SAGE and genomic analyses. The sensitivity of qRT-PCR detection can be 1 tumor cell in 1 mL of blood.

## II. Summary and Explanation

**Vita-Assay™**: The Vita-Assay™ rare cell enrichment (RCE) plate employs the Vitatex proprietary functional cell adhesion matrix (CAM\*: a porous layer of extracellular matrix polymer coated with blood-borne adhesion molecules) that mimics the tumor interstitial micro-environment. The device captures viable, rare cells present in blood or other body fluids for subsequent culture and analyses of the captured cells. The bottom of each well of the Vita-Assay™ plate is coated with a scaffold layer that is comprised of plain or fluorescently labeled CAM<sup>1</sup>. Circulating tumor cells (CTCs: defined using expression of the epithelial cell adhesion molecule [EpCAM] and tumor markers, i.e., CA125 for ovarian tumor; CA19-9 for pancreatic tumor; HER2 for breast tumor; or PSMA for prostate tumor), and their subpopulation named circulating tumor progenitor cells (CTPCs: defined using expression of the cancer stem cell marker CD44v6 and the invasiveness marker seprase) in blood, adhere to the CAM surface due to their high avidity for the extracellular matrix (ECM) and subsequent ingestion of CAM (CAM<sup>+</sup>)<sup>2</sup>. Since the proclivity to degrade and ingest ECM is one of the hallmarks of invasive and metastatic cells, CAM<sup>+</sup> cells captured in Vita-Assay™ plates or chamber slides represent a unique way to identify CTCs and CTPCs.

To capture rare cells from whole blood, anti-coagulated venous blood is subjected either to a red cell lysis (recommended procedure) or density gradient centrifugation (more cell losses were observed). Rare cells, such as CTCs and circulating endothelial cells (CECs) are thus concentrated 1,000-fold into the nuclear cell fraction. Rare cells from the nuclear cell fraction are subsequently enriched by their avidity to CAM scaffolds further through a 18 hour, 37°C incubation in the Vita-Assay™ device. After incubation, unattached nuclear cells are washed away from the CAM surface leaving rare cells and a very small number of white blood cells adhering and invading into the CAM scaffold. Once washed, attached rare cells can be either analyzed directly for their phenotypes or released from CAM scaffolds by enzymatic digestion, and the identity of the enriched rare cells characterized by multi-parameter flow cytometry and microscopy. Alternatively, DNA, RNA and protein of the rare cells dissociated from CAM scaffolds and concentrated in a small volume of cell pellet can be extracted with appropriate buffers for molecular analysis of cancer or other diseases.

An important feature of Vita-Assay™ is that tumor progenitor cells captured can be continuously cultured in the same device for three days. In addition, rare cells captured by

\* US Patents [7,785,814](#), [7,687,241](#), [7,374,898](#), [7,250,492](#); U.S. Patent Application numbers: 10/220,347, 11/010,122, 10/978,029; and international patents and applications covering European, Australia, Canada, Japan and China.

<sup>1</sup> Vita-Assay™ coated with plain CAM (Vita-Assay™ AN6W), red fluorescently labeled CAM (Vita-Assay™ AR6W), and green fluorescent CAM (Vita-Assay™ AG6W) are available.

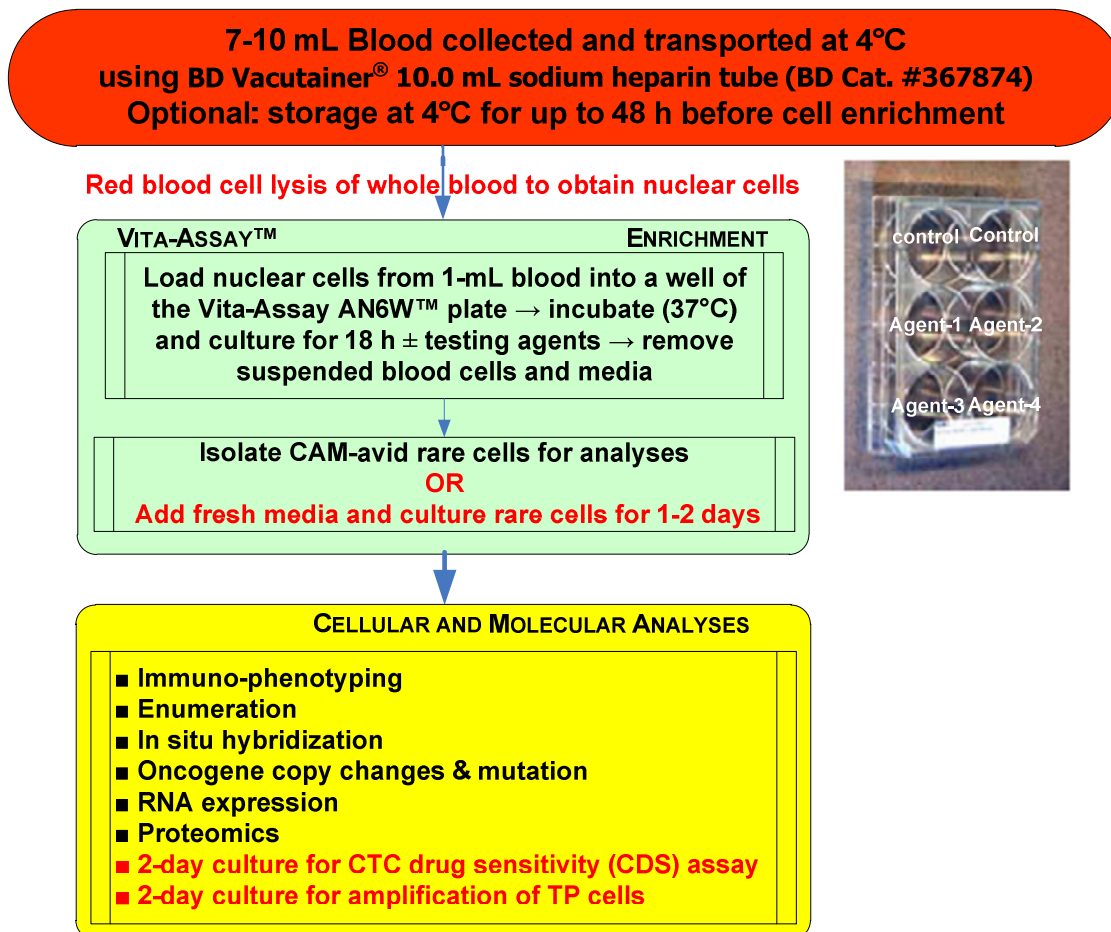
<sup>2</sup> Invasive tumor cells can be identified as CAM<sup>+</sup> only if CAM is fluorescently pre-labeled with red fluorescent dyes (series R) or green fluorescent dyes (series G).

**Vita-Assay™ AN6W plate**

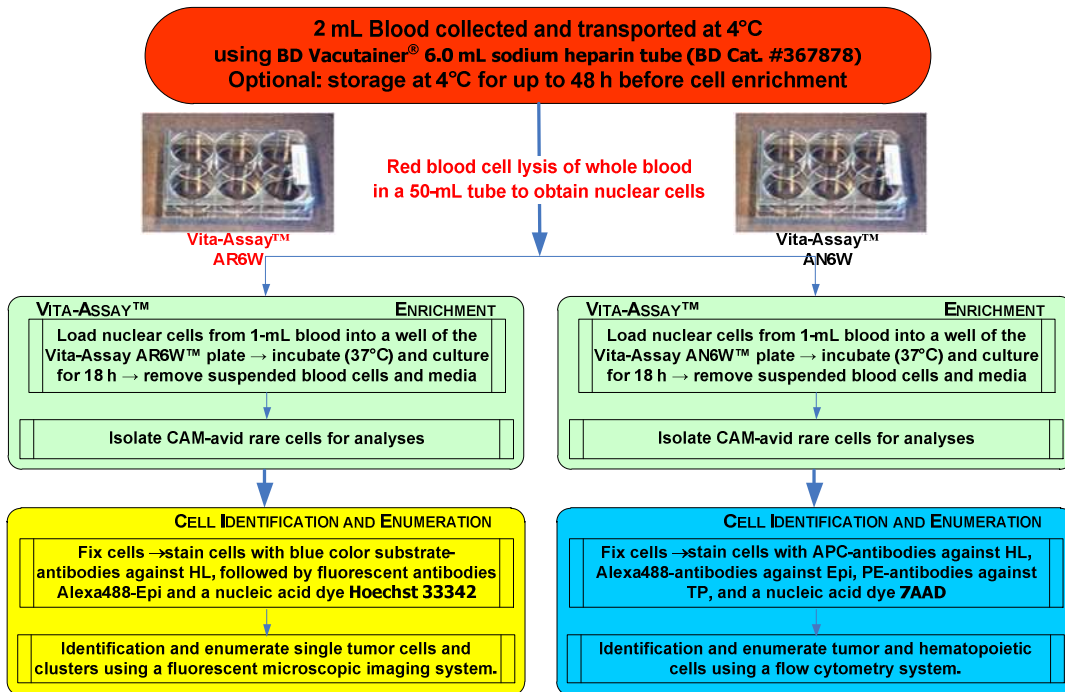
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CAM may be identified directly by their ability to ingest (fluorescently labeled) CAM, and sorted to greater than 90% purity using fluorescence activated cell sorting (FACS).

**Figure 1: Workflow for Vita-Assay™ enrichment of CTCs in blood and downstream applications in cellular and molecular assays.**



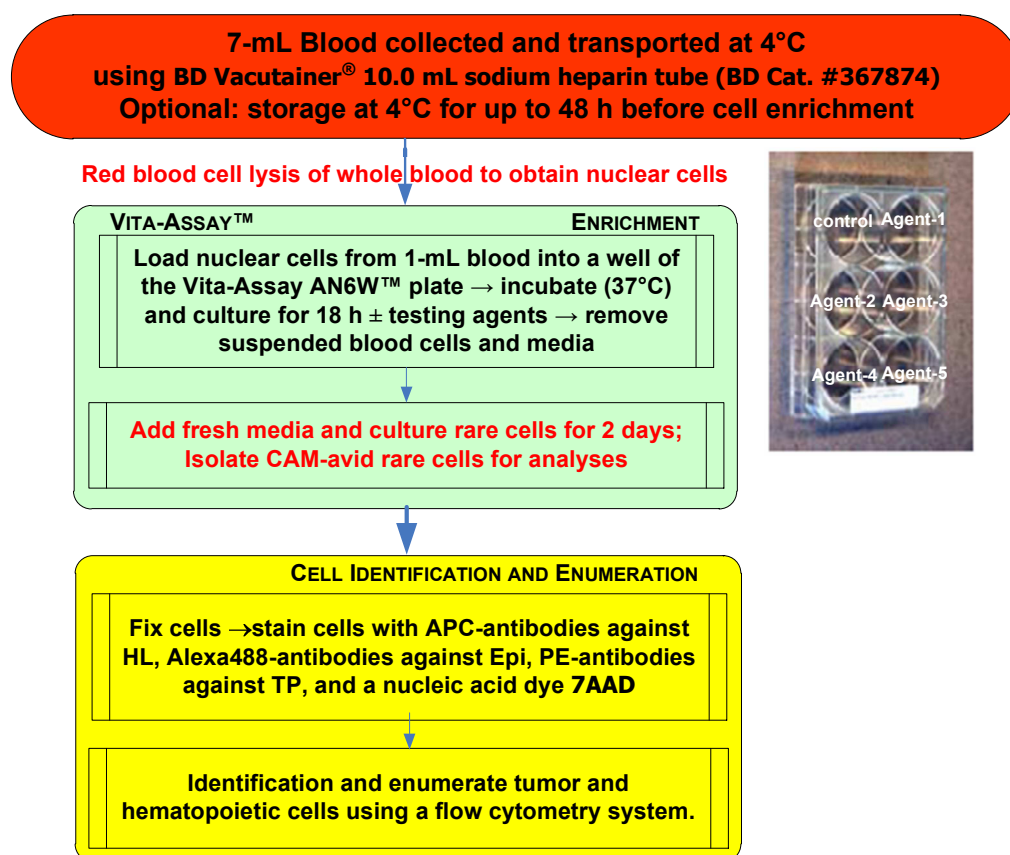
**Figure 2: Workflow of Vita-Assay™ for routine CTC detection using microscopy and flow cytometry.**



**Vita-Assay™ AN6W plate**

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**Figure 3: Workflow of Vita-Assay™ for CTC drug sensitivity (CDS) testing *in vitro*.**



### III. Materials Supplied

- One plate of Vita-Assay™ AN6W, Vita-Assay™ AR6W, or Vita-Assay™ AG6W, respectively, is supplied in each box.

**NOTE:** one Vita-Assay™ AN6W plate can separate rare cells from other blood cells in 1-mL blood per well of the 6-well plate (total of 6-mL blood can be processed using one plate).

- One tube of **Cell Releasing CAM Enzyme**, for enzymatic digestion of CAM and release of progenitor cells, is supplied in each box.

**NOTE:** Vitatex CAM Enzyme must be stored in a freezer (-20°C) immediately upon receipt. Immediately prior to use, dilute the frozen enzyme by adding 6 mL of 1x phosphate buffered saline (PBS), pH 7.4.

**NOTE:** 1:1 mixture of CAM enzyme solution and Trypsin-EDTA (see below) will be used to elute rare cells adhering on the CAM scaffold. The un-used **Enzyme** working solution can be stored at 4°C for a few weeks.

### IV. Additional Materials Required but Not Supplied

**Cell Isolation Procedures:**

- For collection of anti-coagulated venous blood, BD Vacutainer® 10.0 mL sodium heparin tube (BD catalog # 367874) is recommended; others such as BD Vacutainer® 6.0 mL sodium heparin tube (BD catalog # 367878) or 6.0 mL lithium heparin tube (BD catalog # 367886) are also workable.
- For preparation of nuclear cells, red cell lysis buffer (154mM NH<sub>4</sub>Cl, 10mM KHCO<sub>3</sub>, 0.1mM EDTA) pH 8.0 to be mixed with whole blood at a ratio of 1:25 and at 20-30°C. Commercial products currently available include RBC Lysis Buffer 10X (BioLegend catalog # 420301), RBC Lysis Buffer 10X (Imgenex catalog # 10089), and RBC Lysis Buffer (StemCell Technologies catalog # 07850).
- Low-speed, swing-out bucket centrifuge for pelleting cells.
- Sterile 15 mL polystyrene conical centrifuge tubes, i.e., BD cat. #352095.
- A temperature-controlled CO<sub>2</sub> incubator allowing maintenance of human cells in Vita-Assay™ chamber slides or plates.
- Complete cell culture (CCC) medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Cellgro catalog # 10-013-CV) and RPMI 1640 (Cellgro catalog # 10-040-CV) supplemented with 10% calf serum (Hyclone, Thermo Scientific catalog # SH30072.03), 5% Nu-serum (BD catalog # 355504), 2 mM L-glutamine (Cellgro catalog # 25-005-CI), 1 unit/ml penicillin and 10 µg/ml streptomycin (Invitrogen catalog # 15140-122).
- Sterile 1x phosphate buffered saline (PBS), pH 7.4.
- Sterile 0.2% BSA (bovine serum albumin, Sigma catalog # A9085) in 1x PBS, pH 7.4.
- Trypsin-EDTA solution, i.e., Invitrogen catalog # 25200056, 1:1 mixture of CAM enzyme solution and Trypsin-EDTA will be used to elute rare cells adhering on the CAM scaffold.
- A vacuum aspirator for gently removing supernatant waste.

**Cellular analyses using fluorescence microscopy and flow cytometry:**

- Antibody reagents are used for staining of epithelial tumor (Epi) cells, tumor progenitor (TP) cells and hematopoietic lineage (HL) cells, i.e., see publications (Lu et al., 2010; Fan et al., 2009; Paris et al., 2009).

**NOTE:** Vitatex has produced antibody reagent sets available for staining CTCs and visualized by fluorescence microscopy. These include:

- Mouse anti-human epithelial tumor cell surface antigens (Epi), including EpCAM and ESA, catalog # **MABS2002**. Alexa488-conjugated mouse anti-human Epi, catalog # **MABS2002Alexa488**.

- Mouse or rat anti-human tumor progenitor cell surface antigens (TP), including CD44v6 and seprase, catalog # **MABS2003**. Alexa488-conjugated rat anti-human TP, catalog # **MABS2003Alexa488**.
- Mouse anti-human WBCs (CD45 antigens).
- DAPI or Hoechst 33342 for staining cell nuclei.
- Fixative Solution: 1x PBS with 6% paraformaldehyde, pH 7.4.
- Blue color signals for human hematopoietic lineage (HL) or WBCs can be generated using secondary reagent kits obtained through BCIP/NBT (DAKO catalog # K0598 & # K0674, DAKO, Carpinteria, CA).
- Cytospin devices, i.e., StatSpin cytofuge and Filter Concentrators, to concentrate isolated and stained rare cells to 7mm diameter area for microscopic analysis.

**NOTE:** Alternatively, isolated rare cells can be mounted on polylysine-coated glass cover slip, stained with antibodies / DAPI or Hoechst 33342, and mounted on a microscopic slide.

- Mounting medium to mount stained cell samples with glass cover slip on a microscopic slide.
- Fluorescent microscope.

**NOTE:** Vitatex has generated antibody reagent sets for staining CTCs and subsequent counting by flow cytometry. These include:

- Mouse anti-human epithelial tumor cell surface antigens (Epi), including EpCAM and ESA, catalog # **MABS2002**. Alexa488-conjugated mouse anti-human Epi, catalog # **MABS2002Alexa488**.
- Mouse or rat anti-human tumor progenitor cell surface antigens (TP), including CD44v6 and seprase, catalog # **MABS2003**. PE-conjugated rat anti-human TP, catalog # **MABS2003PE**.
- Mouse anti-human hematopoietic lineage (HL) or WBCs (CD45). APC-conjugated mouse anti-human WBCs (CD45 antigens).
- 7-aminoactinomycin (7AAD: Molecular Probe, catalog # A-1310) for staining cell nuclei.

**NOTE:** (a) Preparation of 7AAD stock solution – add 500 µL DMSO into 1 tube containing 1 mg 7AAD powder, vortex, divide into 25 µL aliquots in new sterile 1.5 mL tubes, and store stock solution tubes in a -20°C freezer. The stock solution in each 1.5 mL tube contains 50 µg 7AAD in 25 µL DMSO. (b) Preparation of 7AAD working solution – the 7AAD working solution is made by adding 975 µL PBS into each stock solution tube. 10 µL of 7AAD working solution is sufficient for staining cellular nuclei in one sample. The 7AAD working solution can be stored in a refrigerator for one week.

**NOTE:** Other nucleic acid dyes such as DAPI can be used to replace 7AAD.

- BD FACS Lysing solution, 10x (BD catalog # 349202).
- Polystyrene Tube with Cell-Strainer Cap, 5 mL (BD catalog # 352235).

- A flow cytometer.

### **RNA, DNA or Protein Extraction Procedures:**

- RNA extraction.
  - Commercial RNA extraction kit such as RNeasy Mini Kit (Qiagen Inc., catalog # 74104, [www.qiagen.com](http://www.qiagen.com)).
  - DNA extraction.
    - Commercial Wizard DNA Purification Kit (Promega, Madison, WI) for gene copy number changes of the isolated CTCs, as described (Lu et al., 2010; Fan et al., 2009; Kennedy et al., 2009; Paris et al., 2009). Another DNA isolation kit tested was QIAamp DNA Mini Kit (Qiagen Inc., catalog # 51304, [www.qiagen.com](http://www.qiagen.com)).

## **V. Vita-Assay™ - Cell Isolation Protocol:**

1. **Specimen Collection:** Using your institution's recommended procedure for blood standard venipuncture, collect blood into one or more BD Vacutainer® 10.0 mL sodium heparin tube (BD catalog # 367874). Transport tubes to laboratory.

**NOTE:** Blood in collection tubes may be stored for up to 48 h at 4°C prior to rare cell enrichment steps, although internal evaluation showed that, when blood was stored at 4°C for five days, ~40% of CTCs were recovered.

**NOTE:** All pipetting steps from this point should be conducted in a laminar flow hood using sterile technique.

2. **Preparation of the nuclear cell fraction by red blood cell lysis:** For preparation of nuclear cells from whole blood, we recommend using the following procedure. Red blood cells in the blood samples will be lysed by mixing blood and red cell lysis buffer at a ratio of 1:25 at 20-25°C.
  - 2.1. For a 2-mL blood sample, mix 2-mL blood and 50-mL RBC Lysis Buffer at 20-25°C in a sterile 50-mL conical tube.
  - 2.2. Rotate the 50-mL tube with blood mix on a rotator capable of horizontal rotation of tubes at low speed (10 rpm) for 5 minutes at 20-25°C.
  - 2.3. Pellet nuclear cells by centrifugation to 350xg (1,000rpm) for 5 minutes. Remove supernatant.
  - 2.4. Add 4-mL of CCC medium to each 50mL conical tube to resuspend the cell pellet, resulting in a total of 4-mL cell suspension in CCC medium that were derived from 2-mL whole blood.
3. **Loading cell suspension into wells of Vita-Assay™ AN6W plates:** Aliquot 2-mL of the cell suspension into each of the 6 wells of the Vita-Assay™ AN6W plate coated with

plain CAM. Culture cells in a 5-7% CO<sub>2</sub> incubator at 37°C for 18 h to allow adherence and invasion of tumor cells to CAM.

4. **Washing away floating cells:** After incubation, remove unattached cells with medium into a waste container. Wash wells three times by pipetting 2-mL of 1x PBS into each well each time, followed by moving the plate in horizontal circle three (3) times and discarding wash solution.

**NOTE:** The 6 wells can be used for a simple experiment (by combining all cells collected) or six (6) experimental points (i.e., in testing the responsiveness of cells treated with 6 doses of an anti-cancer drug or 5 drugs and control).

**NOTE:** Adherent cells can be continued culture by adding 1 mL CCC medium into each well (see Section VI below).

#### 5. **Enzymatic release of tumor cells captured by the CAM:**

- a.) Dissolve Vitatex CAM Enzyme by adding 6-mL of 1x PBS, pH 7.4. Prepare 1:1 mixture consisting of 6-mL CAM enzyme and 6-mL Trypsin-EDTA solution (a total of 12-mL).
- b.) Add 1-mL of the diluted enzyme solution into each washed Vita-Assay™ well, and place in a CO<sub>2</sub> incubator at 37°C for 10 minutes to dissolve CAM and release tumor cells into suspension.
- c.) Transfer cell suspension into a new 15 mL conical centrifuge tube. Wash the well (sequentially other wells of the same experimental condition) of Vita-Assay™ plate one (1) time with 3-mL of CCC medium and transfer the wash into the 15 mL tube with enzymatically released cells.

**NOTE:** In case of combining all cells collected, the 6 wells can be sequentially washed with 3-mL of CCC medium and transfer the wash into the 15 mL tube.

- d.) Concentrate cells by centrifuging the 15 mL conical tube at 350xg for 5 min and remove supernatant by gentle aspiration, retaining the last 100 µL containing the enriched cell fraction. The 100 µL cell suspension can be processed for cellular analyses such as microscopy (Section VIII below) and flow cytometry (Section IX below).
- e.) Wash collected cells one time with 10 mL PBS, concentrate cells by centrifuging the 15 mL conical tube at 350xg for 5 min and carefully aspirate the supernatant so that <10 µL containing enriched tumor cells remains, if cell lysis for RNA, DNA or protein is required (Section VI below).

**NOTE:** Aspiration of the supernatant may cause cell loss. On the other hand, incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. These effects may reduce RNA yield.

## VI. Cell Culture Protocol

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6. **For continued culture:** From Step 4 above, remove the PBS wash from each well.
7. **Feed cells captured by the CAM with fresh CCC medium every two days:**
- Add 1-mL of CCC medium into each well, and place the plate in a 5-7% CO<sub>2</sub> incubator at 37°C for two days.
  - Remove the medium with floating cells (by moving the plate in horizontal circle three (3) times and discarding wash solution) and replace with fresh CCC medium.

**NOTE:** Although tumor cells have been cultured in the same Vita-Assay™ plate for more than two months, cultured cells could retain the majority of their genotype and phenotype in CCC medium within two (2) days.

## VII. Microscopy Protocol

8. **Staining with antibody conjugates and nucleic acid dye:** From Step 5-d.) above, loosen the cell pellet thoroughly by flicking the tube.
- Fixation:** Fixation can be done by adding 100 µL of 6% paraformaldehyde in PBS, pH 7.3, into the 100 µL cell suspension, and incubated at 20-25°C for 10 min. Add 3-mL of PBS containing 0.2% BSA, mix and concentrate cells by centrifuging the 15 mL conical tube at 350xg or 1,000 rpm for 3 min, and remove supernatant by gentle aspiration, retaining the last 100 µL containing the enriched cell fraction. [Fixed cell suspension could be stored at 4°C at this point.]
  - Antibody and nucleic acid dye staining:** Add antibody reagents<sup>3</sup> and Hoechst 33342 stock solution<sup>4</sup>, and stain cells at 20-25°C in the dark for 30 min.
  - Washing of stained cells:** Add 3-mL of sterile PBS containing 0.2% BSA. Collect cells by centrifugation at 350xg or 1,000 rpm for 3 min. Remove supernatants and save the last 200-µL containing fixed, stained cell suspension in sterile PBS containing 0.2% BSA.

**NOTE:** Staining for cytokeratins requires treatment of fixed cells with detergents to allow antibodies access to intracellular antigens. However, permeabilization of cell membranes with detergents often cause loss of cell surface antigens that render the reduction of stain signals for cell surface antigens in multiplex settings.

9. **Cytospin preparation of the fixed, stained cells:** To concentrate isolated rare cells to 7mm diameter area for microscopic analysis, cytospin preparation will be performed using devices, i.e., StatSpin cytofuge and Filter Concentrators.

<sup>3</sup> Primary staining uses dilution of 8 µL stock antibody solutions against hematopoietic lineage (HL) markers to 100 µL working solution in PBS containing 0.2% BSA, followed by staining secondary antibody conjugates linked to color substrates. After 1x PBS wash, cells then stained with either 8 µL stock TRITC-antibodies against tumor progenitor (TP) markers and 8 µL stock Alexa488-antibodies against epithelial tumor (Epi) markers in 100 µL working solutions in PBS containing 0.2% BSA.

<sup>4</sup> For microscopic measurement of cellular nuclei: Hoechst 33342 is used to stain nucleic acid of all fixed cells.

**NOTE:** Alternative means of preparing cell samples for microscopy is to mount cell suspension on a slide or circular coverglass coated with polylysine, followed by drying on a 45-60°C hot plate for 30 min.

**10. Preparation for microscopic examination:** Place a drop of mounting medium into cell sample and mounted on a microscopic slide. Examine, record the images and count specific cell types using a fluorescence microscope.

## VIII. Flow Cytometry Protocol

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**11. Staining with antibody conjugates and nucleic acid dye:** From Step 5-d.) above, loosen the cell pellet thoroughly by flicking the tube.

- a.) **Fixation:** Fixation can be done by adding 100 µL of 6% paraformaldehyde in PBS, pH 7.3, into the 100 µL cell suspension, and incubated at 20-25°C for 10 min. Add 3-mL of PBS containing 0.2% BSA, mix and concentrate cells by centrifuging the 15 mL conical tube at 350xg or 1,000 rpm for 5 min, and remove supernatant by gentle aspiration, retaining the last 100 µL containing the enriched cells. [Fixed cell suspension could be stored at 4°C at this point.]
- b.) **Antibody and nucleic acid dye staining:** Add antibody reagents<sup>5</sup> and 7AAD stock solution<sup>6</sup>, and stain cells at 20-25°C (only un-fixed live cells should be stained on ice) in the dark, for 30 min.
- c.) **Washing of stained cells:** Add 3-mL of sterile PBS containing 0.2% BSA. Collect cells by centrifugation at 350xg or 1,000 rpm for 3 minutes. Remove supernatants and save the last 500-µL containing fixed, stained cell suspension in sterile PBS containing 0.2% BSA.

**12. Preparation for flow cytometric cell counting:** Particulates in fixed, stained cells must be filtered away using a Polystyrene Tube with Cell-Strainer Cap (BD catalog # 352235). Collect cells by centrifugation at 1,000 rpm for 3 min. Count cells with a flow cytometer.

## IX. RNA Extraction Protocol

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**13. Disrupt the cells by adding Buffer RLT:** From Step 5-e.) above, loosen the cell pellet thoroughly by flicking the tube. Add 350 µL of Buffer RLT, and vortex or pipet to mix.

**NOTE:** Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

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<sup>5</sup> Add 8 µL Alexa488-antibodies against epithelial tumor (Epi) markers; 8 µL PE-antibodies against tumor progenitor (TP) markers; 30 µL APC-antibodies against hematopoietic lineage (HL) markers, to the 100 µL cell suspension.

<sup>6</sup> For flow cytometric measurement of cellular nuclei: 7AAD is used to stain nucleic acid of all fixed cells.

**14. RNeasy spin columns and RNA extraction procedures according to manufacturer's instruction** [RNeasy Mini Kit (Qiagen Inc., catalog # 74104, [www.qiagen.com](http://www.qiagen.com))].

## **X. NOTES of using Vita-Assay AR6W and AG6W: Identification / Enumeration CTCs with CAM uptake.**

Based on our recent publications (Lu et al., 2010; Fan et al., 2009; Kennedy et al., 2009; Paris et al., 2009) and Vitatex internal R&D, rare cells in the blood of cancer patients are enriched using Vita-Assay™ RCE™ plates and characterized by fluorescence microscopy. CTCs are nucleic acid (NA) and epithelial / tumor markers (Epi) positive but hematopoietic lineage (HL) markers negative –  $NA^+ / EpCAM^+CA125^+^7 / CD45^-$ . Circulating tumor progenitor cells (CTPCs) are CTCs that exhibit CAM uptake ( $CAM^+$ ) or tumor progenitor (TP) markers ( $CD44v6^+SEP^+$ ) –  $NA^+ / EpCAM^+CA125^+ / CD45^- / CAM^+$  or  $CD44v6^+SEP^+$ . Technically, **CTCs are identified using the following criteria.**

- (1). Positive selection procedure for cellular nuclei using the nucleic acid dye Hoechst 33342 to exclude contaminating non-cellular particulates and platelets;
- (2). Positive staining for Alexa488 (or fluorescein)-conjugated antibodies against the epithelial lineage marker EpCAM / ESA and the tumor marker CA125, PSMA, HER2 or CA19-9 (Epi);
- (3). Negative selection procedure using blue color substrate-conjugated antibodies against the hematopoietic lineage (HL) markers, including leukocyte common antigen CD45.

### **CTPCs are identified using the following criteria.**

- (1). Positive selection procedure for cellular nuclei using the nucleic acid dye Hoechst 33342 to exclude contaminating non-cellular particulates and platelets;
- (2). Positive staining for Alexa488 (or fluorescein)-conjugated antibodies against the epithelial lineage marker EpCAM / ESA and the tumor marker CA125, PSMA, HER2 or CA19-9 (Epi);
- (3). Negative selection procedure using blue color substrate-conjugated antibodies against the hematopoietic lineage (HL) markers, including leukocyte common antigen CD45.
- (4). Positive red fluorescence from ingested and concentrated TRITC-labeled collagen fragments ( $CAM^+$ ; the proclivity to degrade and ingest ECM is one of the hallmarks of invasive and metastatic cells); OR Positive staining for Alexa546 (or TRITC)-conjugated antibodies against epithelial stem cell marker CD44v6 and invasiveness marker seprase (SEP).

## **V. The following shows modifications of steps 1 – 10 of Vita-Assay™**

<sup>7</sup> Tumor marker CA125 is used for ovarian cancer samples; PSMA (FOLH1) for prostate cancer; HER2 for breast cancer; CA19-9 for pancreatic cancer and other GI cancers. CA125 is used throughout the text when a tumor marker is used to combine with other markers.

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**cell isolation / identification protocol described above.**

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**Vita-Assay™ AR6W**

Step 3 – use **Vita-Assay™ AR6W** instead of Vita-Assay™ AN6W.

Step 8-b.) – In two separate and parallel cell suspensions, cells will be stained either (1) primary staining uses dilution of 8 µL stock antibody solutions against hematopoietic lineage (HL) markers to 100 µL working solution in PBS containing 0.2% BSA, followed by staining secondary antibody conjugates linked to color substrates. After 1x PBS wash, cells then stained with 8 µL stock Alexa488-antibodies against tumor progenitor (TP) markers in 100 µL working solutions in PBS containing 0.2% BSA, or (2) primary staining uses dilution of 8 µL stock antibody solutions against hematopoietic lineage (HL) markers to 100 µL working solution in PBS containing 0.2% BSA, followed by staining secondary antibody conjugates linked to color substrates. After 1x PBS wash, cells then stained with 8 µL stock Alexa488-antibodies against epithelial tumor (Epi) markers in 100 µL working solutions in PBS containing 0.2% BSA.

**Vita-Assay™ AG6W**

Step 3 – use **Vita-Assay™ AG6W** instead of Vita-Assay™ AN6W.

Step 8-b.) – In two separate and parallel cell suspensions, cells will be stained either (1) primary staining uses dilution of 8 µL stock antibody solutions against hematopoietic lineage (HL) markers to 100 µL working solution in PBS containing 0.2% BSA, followed by staining secondary antibody conjugates linked to color substrates. After 1x PBS wash, cells then stained with 8 µL stock TRITC-antibodies against tumor progenitor (TP) markers in 100 µL working solutions in PBS containing 0.2% BSA, or (2) primary staining uses dilution of 8 µL stock antibody solutions against hematopoietic lineage (HL) markers to 100 µL working solution in PBS containing 0.2% BSA, followed by staining secondary antibody conjugates linked to color substrates. After 1x PBS wash, cells then stained with 8 µL stock TRITC-antibodies against epithelial tumor (Epi) markers in 100 µL working solutions in PBS containing 0.2% BSA.

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**VI. Discussion: Specimen Collection, Rare Cell Enrichment and Short-Term Culture**

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Potential applications of short-term culture of CTCs are to: (1) amplify the number of CTCs in a specific blood sample within three (3) days, (2) characterize the genotype and phenotype of these cultured cells by molecular analytic methods, (3) store the amplified cells in a -170°C freezer, so that researchers can have a second look on these cells, i.e., the responsiveness to anti-cancer agents, a strategy moving forward personalized medicine, and (4) effectively perform CTC drug sensitivity (CDS) testing to examine the responsiveness of CTCs and TP cells to anti-cancer agents.

**NOTE:** The methods and procedures described here can be applied to the amplification of circulating endothelial cells and circulating endothelial progenitor cells in whole blood, as well as different rare cells in bone marrow.

## References

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